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REVIEW

Physiologic Responses to Hypoxia and Implications for Hypoxia-Inducible Factors in the Pathogenesis of Rheumatoid Arthritis

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Introduction

Oxygen homeostasis represents a basic principle for all higher organisms. Reduction of the normal oxygen concentrations causes a metabolic demise, because in mitochondrial oxidative phosphorylation, oxygen is the terminal electron acceptor during ATP formation. In addition, several enzymatic reactions require oxygen as a substrate. In contrast, increased oxygen concentrations bear the risk of oxidative damage to proteins, lipids, and nucleic acids. Therefore, even slight changes in systemic and cellular oxygen concentrations induce a tightly regulated machinery of short- and long-acting response pathways, aiming to keep the supply of oxygen within the physiologic range.

In particular, the molecular responses to hypoxia have been elucidated in detail during the past several years. In this context, the cloning and molecular characterization of the transcription factor hypoxia-inducible factor 1 (HIF-1) mark a breakthrough discovery in knowledge of cellular adaptation to reduced oxygenation (1). Under hypoxic conditions, HIF-1 protein accumulates in many different cell types and activates the transcription of genes that are of fundamental importance for oxygen homeostasis, including genes involved in energy metabolism, angiogenesis, vasomotor control, apoptosis, proliferation, and matrix production (2) (Table 1).

However, regulatory mechanisms that are important under physiologic conditions may have disastrous effects when the delicate control of HIF-1 expression is disrupted in pathologic situations. For example, HIF-1 is expressed in a variety of tumors and is thought to be a critical factor in tumor progression (3), because tumor growth is strongly dependent on blood supply. Indeed, it has been demonstrated that HIF-1–induced overexpression of vascular endothelial growth factor (VEGF) drives the initiation of angiogenesis in many solid tumors. When HIF-1–mediated transcription was disrupted in tumor cells, hypoxia-inducible gene expression (including that of VEGF) was found to be attenuated, and vessel density as well as tumor growth were remarkably reduced compared with that in control tumor cells (4,5).

The present review summarizes current knowledge of the molecular signaling pathways in response to hypoxia. Furthermore, possible links between HIF-1 signaling and the pathogenesis of rheumatoid arthritis (RA) will be discussed.

Molecular structure of HIF-1

In 1995, Wang et al cloned the transcription factor HIF-1, based on its ability to bind to the 3′ enhancer region of the erythropoietin gene (1). Structural analysis revealed that HIF-1 consists of 2 different subunits, HIF-1α (120 kd) and HIF-1β (91–94 kd). Sequence analyses showed that HIF-1α was a novel protein, whereas HIF-1β was identical to the dioxin receptor aryl hydrocarbon receptor nuclear translocator (ARNT). Both subunits contain a basic helix–loop–helix domain, enabling them to recognize and bind to specific DNA sequences, the so-called HIF-1 DNA binding site

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Table 1. Genes regulated by hypoxia-inducible factor 1α at the transcription level*

Functional group/gene product	Ref.
Anaerobic metabolism	64
Hexokinases 1 and 2	64, 119, 120
Phosphofructokinase L	64, 120
Aldolase C	64
Triosephosphate isomerase	64, 120
Pyruvate kinase M	64, 120
Lactate dehydrogenase A	64
Enolase 1	33, 64
Glucose transporter 1	
pH regulation	
Carbonic anhydrase IX	121
Nucleotide metabolism	
Adenylate kinase 3	33
Angiogenesis	
VEGF- and endocrine gland–derived VEGF	64, 199, 122, 123
VEGF receptor 1 (Flt-1)	124
Plasminogen activator inhibitor 1	63
Vascular tonus	
α_{1B}-adrenoreceptor	125
Inducible nitric oxide synthase 2	126
Endothelin 1	26, 127
Heme oxygenase 1	128
Erythropoiesis	
Erythropoietin	129–131
Proliferation and apoptosis	
p21	119
Nip3 (BNIP3)	132, 133
Nip3-like protein X	133
Insulin-like growth factor 2	30
Insulin-like growth factor binding proteins 1, 2, 3	30, 134
Iron metabolism	
Transferrin	32
Transferrin receptor	135
Ceruloplasmin	136
Matrix metabolism	
Prolyl 4-hydroxylase α(I)	137
Collagen type V α(I)	121
Transcription factors	
Dec-1	121
Ets-1	130

* VEGF = vascular endothelial growth factor.

(HBS), within the regulatory regions of hypoxia-inducible genes. Another characteristic feature of both proteins are the PAS regions, which are located at the N-termini and consist of 2 subunits (PAS-A and PAS-B). PAS is an acronym for the first transcription factors in which a PAS domain was identified (Per, Arnt, and Sim). Using HIF-1α deletion mutants, Jiang et al demonstrated that the helix–loop–helix domain and the PAS-A region of HIF-1α are sufficient for heterodimerization with ARNT (6).

The most intriguing structural element of HIF-1α is the oxygen-dependent degradation (ODD) domain, which links HIF-1α to the cellular oxygen sensor. This domain is essential for degradation under normoxic conditions and for stabilization under hypoxic conditions. HIF-1α–induced gene expression is mediated via 2 transactivation domains: one is located at the C-terminus of HIF-1α, whereas the second one overlaps with the ODD domain. Regulation of the second transactivation domain likely occurs as a side effect of the stabilization of HIF-1α, whereas the C-terminal transactivation domain is functionally independent from the ODD domain (for review, see refs. 7–9). The structural elements of HIF-1α are shown in Figure 1.

Stabilization of HIF-1α protein

Whereas the concentration of HIF-1β/ARNT is independent of oxygen levels, cellular HIF-1α is not detectable under normoxic conditions, because HIF-1α is degraded in proteasomes immediately after translation (Figure 2). The levels of HIF-1α increase exponentially after exposure to an oxygen concentration of $<6\%$, with a cell-type–dependent maximal response at a concentration of $\sim 0.5\%$. This corresponds to a partial pressure of O_2 of 10–15 mm Hg. In contrast, stress-inducible transcription factors such as activator protein 1 (AP-1) and nuclear factor κB are induced only at near-anoxic oxygen levels (10,11).

The molecular basis for this oxygen-dependent regulation is hydroxylation of two proline residues at positions 402 and 564 within the ODD domain of HIF-1α (12), which is similar to the prolyl modification of collagens (13,14). A family of 4 HIF prolyl hydroxylase domains (PHDs), designated PHD-containing proteins, has been identified so far, based on their similarity to the *Caenorhabditis elegans* homolog EGL-9 and collagen hydroxylases (15–17). The PHDs are iron-dependent dioxygenases requiring oxygen and oxoglutarate as co-substrates. Binding of oxygen requires vitamin C–dependent maintenance of the iron in the catalytic site as a ferrous ion. PHDs use one of the bound oxygen atoms to hydroxylate proline residues 402 or 564 of HIF-1α, whereas the second oxygen atom is incorporated into oxoglutarate, thereby generating succinate and carbon dioxide. The ability of PHDs to modify HIF-1α is limited by the oxygen concentration. Under normoxic conditions, PHDs can hydroxylate HIF-1α efficiently, leading to the rapid degradation of the HIF-1α subunit, whereas low oxygen levels prevent this reaction. Therefore, members of the PHD family function as an intracellular oxygen–sensing mechanism and provide the molecular basis for the regulation of HIF-1α protein concentrations by cellular oxygen partial pressure (16).

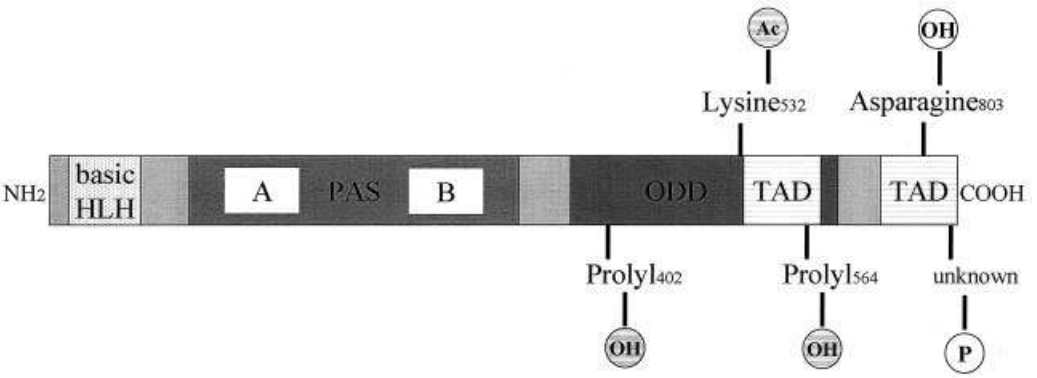

Figure 1. Structural elements of hypoxia-inducible factor 1α (HIF-1α). The basic helix–loop–helix (HLH) domain is essential for DNA binding of HIF-1α. The 2 PAS (Per, Arnt, and Sim) domains of HIF-1α are involved in heterodimerization and DNA binding and might also influence transactivation. HIF-1α bears 2 transactivation domains (TADs), one at the C-terminus and one within the oxygen-dependent degradation (ODD) domain. The ODD domain mediates degradation of HIF-1α under normoxic conditions. Hydroxylation (OH) of proline residues 402 and 564 within the ODD domain of human HIF-1α by prolyl hydroxylases under normoxic conditions promotes binding of the von Hippel-Lindau protein and degradation of HIF-1α; hydroxylation of asparagine residue 803 in the C-terminal transactivation domain at normoxic Po_2 levels decreases the transactivation of HIF-1α–regulated genes. Similar to hydroxylation of proline residues 402 and 564, acetylation (Ac) of lysine residue 532 by ADP-ribosylation factor domain protein 1 leads to degradation of HIF-1α under normoxic conditions. Phosphorylation (P) of unidentified residues has also been shown to regulate transactivation activity of HIF-1α.

Recently, an additional mechanism for the regulation of HIF-1α protein stability was identified (18). Using pull-down assays and deletion constructs, Jeong et al demonstrated that an ADP-ribosylation factor domain protein (ARD1) homolog binds directly to the ODD domain of HIF-1α in the cytoplasm. After binding, ARD1 acetylates a single lysine residue at position 532 in the ODD domain. In vitro ubiquitination assays revealed that only acetylation of lysine 532 (but not other lysine residues) stimulates the rapid degradation of HIF-1α. Mutation of lysine residue 532 or application of antisense ARD1 resulted in stabilization of HIF-1α even under normoxic conditions. The role of ARD1-dependent acetylation was further confirmed by experiments showing that inhibition of deacetylation with sodium butyrate or trichostatin A markedly reduced the accumulation of HIF-1α under hypoxic conditions. As shown by VEGF promoter–driven luciferase reporter gene assays, ARD1 not only destabilizes HIF-1α protein but also down-regulates the transactivation activity of HIF-1α in ARD1-transfected HT1080 cells exposed to hypoxic conditions.

Blockade of hydroxylation of proline residues 402 and 564 as well as blockade of acetylation of lysine 532 have convincingly been demonstrated to prevent degradation of HIF-1α under normoxic conditions, thus abolishing the oxygen-dependent regulation of HIF-1α signaling (13,18,19). These findings suggest that both pathways are essential for the physiologic regulation of the cellular responses to hypoxia. However, many questions have not yet been addressed sufficiently. For instance, it is currently unclear whether the PHD isoforms are functionally redundant and whether other family members might compensate for the loss of one PHD. Experiments in vitro suggest that PHD2 has the highest specific affinity for HIF-1α (20), but the relevance of this finding for the degradation of HIF-1α in specific tissues in vivo is not clear. Moreover, regulation of the expression of PHDs and ARD1 has not been clarified. Interestingly, preliminary data indicate that some members of the PHD family might be induced by hypoxia itself (16). Further studies are needed to show whether differential expression and/or mutations of these enzymes are linked to certain disease manifestations in vivo.

The rapid degradation of HIF-1α under normoxic conditions is mediated by the von Hippel-Lindau (VHL) tumor suppressor gene product pVHL (21). The β subunit of pVHL interacts directly with the ODD domain of HIF-1α if proline residues 402 and 564 are hydroxylated, but not without this modification (Figure 2). The VHL protein itself is part of the E3 ubiquitin ligase complex consisting of elongin B and C, Cul-2, Rbx1, E2, and several other incompletely characterized

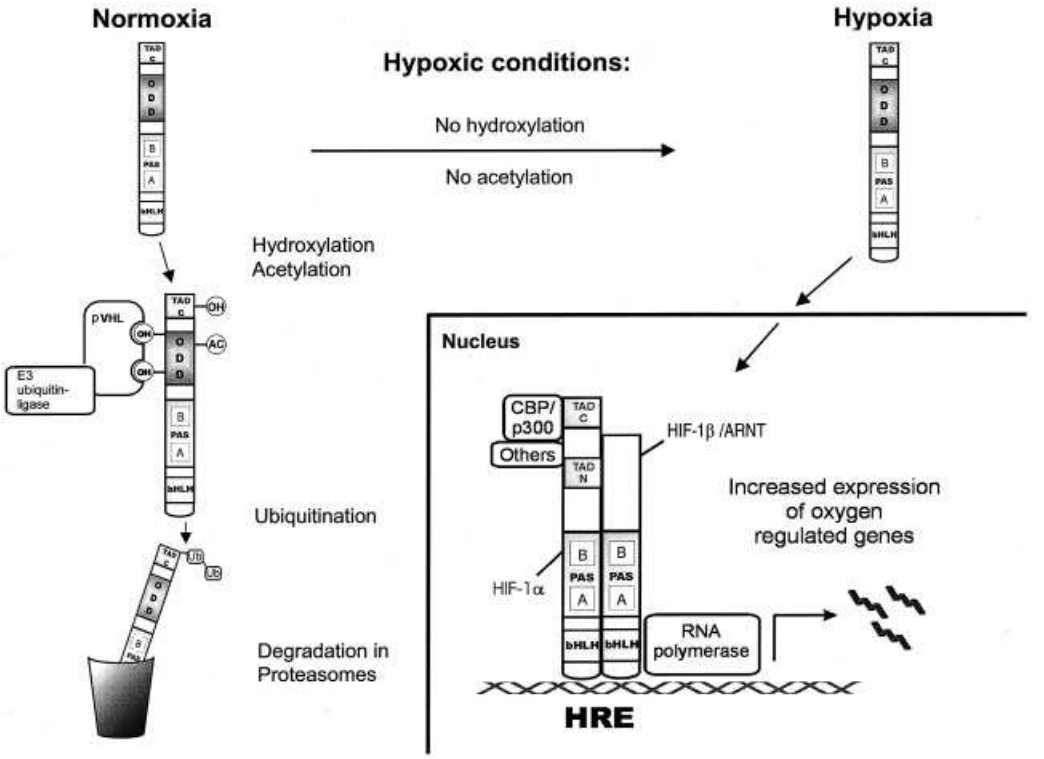

Figure 2. Schematic overview of the regulation and action of HIF-1. Under normoxic conditions, HIF-1α is rapidly degraded. Hydroxylation of proline residues and acetylation of a lysine residue lead to binding of the von Hippel-Lindau protein (pVHL) as part of the E3 ubiquitin ligase complex. Subsequently, the HIF-1α protein undergoes ubiquitination (Ub) and rapid degradation in proteasomes. Under hypoxic conditions, hydroxylation and acetylation do not occur due to the lack of oxygen, the von Hippel-Lindau/E3 ubiquitin ligase complex cannot bind, and the HIF-1α protein is stabilized. Then, HIF-1α is translocated into the nucleus, dimerizes with HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT), and recruits cofactors such as cAMP response element binding protein binding protein (CBP)/p300. Finally, HIF-1 binds to the hypoxia-responsive element (HRE) in regulatory regions of oxygen-regulated genes and increases their transcription. bHLH = basic helix–loop–helix (see Figure 1 for other definitions).

components. Interaction of proline-hydroxylated HIF-1α and pVHL/E3 ubiquitin ligase complex activates the ubiquitination machinery, thereby promoting a signal for the degradation of HIF-1α within the proteasome (13,19). A similar mechanism of recognition is proposed for the acetylated lysine residue 532, because this modification has also been shown to stimulate ubiquitination of HIF-1α (18). Under hypoxic conditions, the ODD domain of HIF-1α is not hydroxylated or acetylated, pVHL cannot bind, and subsequently HIF-1α is not ubiquitinated. This inhibits degradation of HIF-1α in the proteasome, leading to stabilization of the HIF-1α protein.

Nuclear translocation of HIF-1α

Using an HIF–green fluorescent protein (GFP) fusion protein, Kallio et al observed that HIF-1α is translocated into the nucleus at low oxygen concentrations, with a complete nuclear import after ~1 hour of hypoxic exposure at 37°C (22). As with other transcription factors, the nuclear import of proteins larger than 45 kd is an energy-dependent transport process that requires the presence of nuclear localization signals (NLS) within the transported transcription factor. The HIF-1α protein contains 2 NLS motifs, one within the basic helix–loop–helix domain at the N-terminus and a second one at the C-terminus. Transfection of COS-7 cells with different GFP–HIF-1α fusion proteins showed that the C-terminal (but not the N-terminal) NLS motif mediates the hypoxia-inducible nuclear import of HIF-1α, and that the B unit of the PAS domain is involved in the repression of the nuclear import of HIF-1α under normoxic conditions (22). Subsequent analysis revealed that the C-terminal NLS motif of HIF-1α represents a

novel form of a bipartite NLS consisting of 2 basic residues and a spacer of 27 amino acids in between (23). Despite its name implying a function in nuclear translocation of HIF-1α protein, the "nuclear translocator" ARNT is not required for this process (24). Interestingly, normoxic overexpression of HIF-1α via a Tet-Off Expression System (BD Biosciences, San Jose, CA) leads to an accumulation in the nucleus, suggesting that the translocation of HIF-1α into the nucleus is blocked under normoxic conditions by a saturable mechanism rather than stimulated by hypoxia (25). However, the molecular mechanisms responsible for this phenomenon remain unidentified.

Binding of HIF-1 to HIF binding sites, and formation of the transcriptional complex

After translocation into the nucleus, HIF-1α dimerizes with ARNT/HIF-1β (Figure 2). The HIF-1 heterodimer then binds via the basic helix–loop–helix domain to the HBS, which as been characterized as a 5′-RCGTG-3′ motif within the hypoxia-responsive element of most hypoxia-regulated genes (8,9,26). The HBS contains a CpG island, which is well known as a target for methylation. Indeed, methylation of the CpG island within the HBS has also been demonstrated for hypoxia-regulated genes such as erythropoietin, leading to an abrogation of HIF-1 binding and gene activation (27). The HBS is essential, but is not sufficient for gene activation by HIF-1. A complete hypoxia-responsive element contains, besides the HBS, additional binding sites for transcription factors that are not sensitive to hypoxia. These costimulatory factors, including, for example, activating transcription factor 1/cAMP response element binding protein 1 (CREB-1) in the lactate dehydrogenase A gene (28) or AP-1 in the VEGF gene (29), are also required for efficient activation of the transcription of oxygen-sensitive genes. Alternatively, multimerization of HBS can substitute for additional transcription factors in several HIF-regulated genes such as transferrin, phosphoglycerate kinase 1, insulin-like growth factor binding protein 1, and glucose transporter 1 (30–33).

Regulation of HIF-1 transactivation

For efficient induction of HIF-1–regulated genes, HIF-1 needs to be activated. Simple blockade of the degradation of HIF-1α (e.g., with chemical proteasome inhibitors such as *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norvalinal) results in an accumulation of HIF-1α but is not sufficient for transactivation (34). Two modifications of HIF-1α involved in the regulation of its activity have been identified so far, namely, hydroxylation of the C-terminal transactivation domain (Figure 1) and protein phosphorylation after activation of tyrosine kinase receptors (Figure 3).

At low concentrations of oxygen, but not under normoxic conditions, the C-terminal transactivation domain of HIF-1α recruits several coactivators that are required for HIF-1 signaling (5,35). Mass spectrometry revealed that under normoxic conditions the molecular mass of this transactivation domain was 16 daltons greater than under hypoxic conditions (36). Further experiments showed that the increase of molecular mass under normoxic conditions was caused by hydroxylation of a conserved asparagine residue at position 803 in the C-terminal transactivation domain (Figure 1). Mutation of this asparagine residue as well as the lack of hydroxylation lead to a permanent recruitment of cofactors and yield a constitutively active HIF-1α. The enzyme responsible for asparagine hydroxylation has recently been shown to be identical to factor-inhibiting HIF-1 (FIH-1) (36–38). FIH-1 forms ternary complexes with HIF-1α and pVHL, which activate histone deacetylases and hence might counterbalance the changes in the chromatin structure induced by the transcriptional coactivators p160/steroid receptor coactivator 1 (SRC-1) and cAMP response element binding protein binding protein (CBP)/p300. Similar to prolyl hydroxylation within the ODD domain, iron and oxoglutarate are essential for asparagine hydroxylation, and this process is limited by the oxygen partial pressure. Therefore, one might speculate that FIH-1 is part of the O_2-sensing mechanism (39).

Besides hypoxia, a variety of cytokines and growth factors have been demonstrated to be capable of stabilizing and activating HIF-1α under normoxic conditions (Figure 2), at least in cell cultures in vitro. Examples include interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), platelet-derived growth factor, fibroblast growth factor 2, and insulin-like growth factors (40–42). Binding of these ligands to their receptor tyrosine kinases activates, among others, the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt kinase/FK 506 binding protein 12-rapamycin associated protein (FRAP) pathway. Stimulation of different receptor tyrosine kinases in different cell types has been demonstrated to increase the levels of HIF-1α and induce the transcription of oxygen-dependent genes (43). This effect could be inhibited by antagonists of PI 3-kinase and FRAP. Sur-

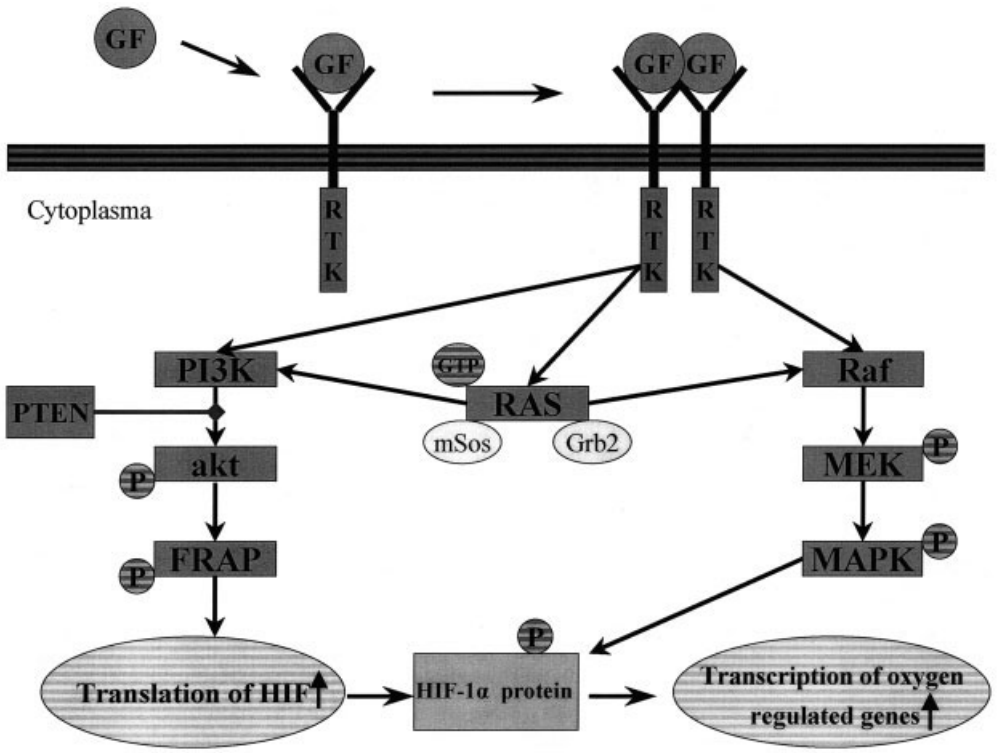

Figure 3. Protein phosphorylation (P) after activation of receptor tyrosine kinases (RTKs). Many growth factors and cytokines such as interleukin-1β, tumor necrosis factor α, transforming growth factor (GF) β, platelet-derived growth factor, fibroblast growth factor 2, and insulin-like growth factors can stimulate the transcription of HIF-1–regulated genes under normoxic conditions. Binding of these cytokines to their cell surface receptors induces homodimerization of the receptors and activates their tyrosine kinases. One of the intracellular signaling pathways that is activated by RTKs is the phosphatidylinositol 3-kinase (PI3K; PI 3-kinase)/Akt kinase/FK 506 binding protein 12-rapamycin associated protein (FRAP) pathway. Activation of RTKs stimulates PI 3-kinase, which in turn phosphorylates Akt (also termed protein kinase B). Akt activates FRAP, leading to an increased translation of HIF-1α that is sufficient to induce transcription of HIF-1α–regulated genes, even under normoxic conditions. The PI 3-kinase/Akt kinase/FRAP pathway is negatively regulated by the tumor suppressor gene PTEN. PTEN encodes for a phosphatase that dephosporylates PI phosphates and thus abolishes activation of Akt and other targets of the PI 3-kinase pathway. Another pathway involved in the regulation of HIF-1 signaling that is activated upon stimulation with growth factors is the MAP kinase (MAPK) pathway. RTKs recruit the inactive cytoplasmic Raf protein to the plasma membrane, where it gets fully activated by phosphorylation and interactions with cationic lipids. Then, Raf binds and phosphorylates mitogen-activated extracellular signal–regulated kinase (MEK). Finally, MEKs activate the MAPKs p42 and p44 (Erk2 and Erk1), which phosphorylate HIF-1α. This enhances the transcriptional activity of HIF-1α and increases expression of HIF-1α–regulated genes. The PI 3-kinase/Akt/FRAP pathway as well as the MAPK pathway can also be stimulated by Ras. Stimulated RTKs can activate the small guanosinetriphosphate (GTP) binding protein Ras via the adaptor molecule Grb2 and the guanine exchange factor mSos. See Figure 1 for other definitions.

prisingly, the accumulation of HIF-1α upon stimulation of the PI 3-kinase/Akt kinase/FRAP pathway is caused by up-regulation of protein synthesis rather than decreased proteasomal degradation of HIF-1α mediated by sequences in the 5′-untranslated region of the HIF-1α gene (43). Signaling via the PI 3-kinase/Akt kinase/FRAP pathway is counterregulated by the tumor suppressor gene PTEN. PTEN protein dephosphorylates the products of PI 3-kinase reactions. Along this line, loss of PTEN in tumors leads to a dramatic increase of HIF-1α protein associated with neoangiogenesis and tumor progression (44). Most interesting is the fact that in RA a lack of PTEN is found at sites of synovial invasion into the cartilage (45).

Cytokines and growth factors can influence the transcriptional activity of HIF-1 also via the mitogen-

activated protein kinase (MAPK) pathway. Upon activation of receptor tyrosine kinases, the serine/threonine protein kinase Raf is activated either by direct phosphorylation or via Ras. Raf activates the mitogen-activated extracellular signal–regulated kinase, which in turn phosphorylates the MAPKs (46). Members of the MAPK family such as p42, p44, p38α, and p38γ can phosphorylate unidentified residues of HIF-1α in vitro (Figure 1), leading to electrophoretic migration properties similar to the modifications of HIF-1α under hypoxic conditions (47,48). Phosphorylation of HIF-1α by MAPKs increases HIF-1α–dependent reporter gene expression, but in contrast to PI 3-kinase/Akt kinase/FRAP signaling, it does not induce translation of HIF-1α protein (49).

Cofactors of HIF-1 and negative feedback pathways

For the induction of oxygen-dependent genes by HIF-1α, binding of general coactivators to the 2 transactivation domains of HIF-1α is required (50,51). This includes members of the p300/CBP and p160/SRC-1 family of coactivators, which bear histone acetyltransferase activity and can remodel the chromatin structure (52). As described in the previous section, hydroxylation of asparagine residue 803 of HIF-1α inhibits binding of the cysteine/histidine-rich domain 1 (CH1) of p300/CBP to the C-terminal transactivation domain (53). Binding of the transcription factor p35srj (previously named melanocyte-specific gene related gene 1 and CITED2) to the CH1 domain of p300/CBP also prevents the interaction of p300/CBP with HIF-1α (53). Interestingly, p35srj is, by itself, a HIF-1 target gene induced by hypoxia, thereby creating a negative feedback loop. Finally, an HIF-1α proteasome-targeting factor (HPTF) was proposed by Berra et al (49), who observed that the half-life of HIF-1α protein after reoxygenation was inversely related to the duration of hypoxic exposure, and that this phenomenon was blocked by inhibition of HIF-1–dependent transcription. Based on these findings, the authors suggested that HIF-1 induces the synthesis of HPTF, which in turn activates the HIF-1α degradation pathway after reoxygenation. However, the molecular structure of HPTF has not been identified. Because the expression of PHDs might be induced by hypoxia, further studies must analyze whether HPTF might be identical to PHD family members.

Other members of the HIF family

Two proteins closely related to HIF-1α have been identified and designated HIF-2α (54,55) and HIF-3α (56,57). HIF-2α is also referred to as endothelial PAS domain protein 1 (58), HIF-α–like factor (54), and HIF-related factor (55). HIF-1α and HIF-2α are ubiquitously expressed, whereas HIF-3α shows a more restricted pattern, with high expression levels in the brain, thymus, and muscle (57,59,60). HIF-2α is similar to HIF-1α with regard to genomic organization, protein structure, dimerization with ARNT, DNA binding, and transactivation (21,35,61,62). Furthermore, the expression patterns of HIF-1α and HIF-2α are partially overlapping, and an accumulation of both proteins can be observed under hypoxic conditions (59,60,63). Therefore, one might speculate that one α subunit could compensate for loss of the other one. However, experiments with knockout mice demonstrated that HIF-α subunits are not functionally redundant, because targeting of either one of the HIF-α subunits results in embryonic lethality, and one subunit cannot compensate for the loss of the other subunit (31,64,65).

Recently, a novel splicing variant of HIF-3α, which inhibits HIF-mediated gene expression, was discovered (66). This antagonist, called the inhibitory PAS domain protein (IPAS), shows high structural similarity to the basic helix–loop–helix domain and to a lesser extent to the PAS region of HIF-1α and HIF-1β, which are necessary for dimerization. However, IPAS lacks the C-terminal region of HIF-1α and HIF-1β, which harbors the transactivation domains. IPAS is expressed at high levels in corneal epithelium cells, where it competes with HIF-1β/ARNT to form complexes with HIF-1α. Because the IPAS/HIF-1α complex does not bind to HIF-1 DNA binding sites and does lack transactivation activity, the overexpression of IPAS prevents HIF-1–mediated neoangiogenesis in the cornea. Application of IPAS antisense oligonucleotides to the cornea, a physiologically hypoxic tissue, induced angiogenesis via an upregulation of VEGF by HIF-1. Similarly, stable overexpression of IPAS in hepatoma cells inhibited tumor growth due to a reduced vascular density (66).

Three other inhibitors of HIF-1 have been discovered, but their physiologic relevance is less clear. Chun et al detected 2 alternative splicing variants of HIF-1α messenger RNA (mRNA) in 293T cells: the first one, lacking exon 12, is induced by zinc and was therefore designated HIF-1αZ (67). In the other isoform, HIF-1α^{516}, which is constitutively expressed in 293T cells, exons 11 and 12 are missing (68). Both of the resulting proteins are unable to induce transcription of HIF-1–regulated genes, but they are still able to compete with HIF-1α for dimerization with ARNT and DNA binding sites. Furthermore, a natural antisense

transcript, complementary to the 3′-untranslated region of HIF-1α mRNA, was reported (69).

Lessons from knockout experiments: physiologic functions of HIFs

To further analyze the physiologic functions of HIFs, gene targeting of HIF-1α and HIF-2α has been employed. Mice containing one wild-type and one mutant allele for HIF-1α show no morphologic defects, develop normally, and their acute responses to hypoxia are also normal (70,71). However, the mechanisms for adaptation to chronic hypoxia are impaired, with delayed development of polycythemia and impaired hypoxia-induced vascular remodeling with reduced pulmonary hypertension and reduced right ventricular hypertrophy. Neuronal cells from the carotid body, a major hypoxia sensory organ, showed reduced activity at low oxygen levels (70,71).

Mice lacking both alleles for HIF-1α are not viable and die at mid gestation (approximately embryonic day 10.5) (31,64,72). These mice show a wide range of neuronal malformations, including failure of neural tube closure, prolapsed neural folds, and cystic enlargement of the hindbrain, probably due to the profound loss of supporting mesenchymal cells within these regions. In addition, cardiovascular defects have been observed in all HIF-1α knockout embryos. The brachial arch vessels are either severely hypoplastic or absent, and localized regions of the dorsal aorta are dilated. The vascular endothelium is discontinuous and attenuated. Furthermore, abnormal cellular proliferation results in hyperplasia of the presumptive myocardium and a subsequent reduction of the size of the ventricular cavity and of the outflow tract.

Growth plate chondrocytes in the developing bone undergo well-controlled phases of cell proliferation, differentiation, and apoptosis, finally resulting in the replacement of the cartilage matrix with trabecular bone matrix. As analyzed by injection with a marker for bioreductive activity (EF-5), there is a physiologic gradient of oxygenation from the outer to the inner region of the developing growth plate, which correlates with the expression of HIF-1α (73). Conditional knockout mice, lacking HIF-1α specifically in the cartilaginous growth plate, showed massive cell death in the center of both the proliferative and hypertrophic zones, leading to gross skeletal malformation, with shorter hind and fore limbs. These data indicate that HIF-1α is critical for the survival of cells in a fully differentiated tissue under hypoxic conditions. Moreover, epiphyseal chondrocytes lacking HIF-1α are unable to maintain levels of ATP under hypoxic conditions, indicating that HIF-1α is essential for the regulation of chondrocyte metabolism, probably via an increase in glycolysis. In fact, expression of extracellular matrix proteins such as aggrecan and type II collagen is decreased in HIF-1α null chondrocytes compared with wild-type chondrocytes (74).

Studies of placentas from ARNT knockout mice revealed that HIF-1 is also essential for placentation (75). Under hypoxic conditions, as found in the uterus in vivo, cultured cytotrophoblasts proliferate, whereas in a normoxic environment they lose this ability and differentiate (76). Based on experiments with HIF-1 antisense oligonucleotides in human chorionic villous explants, it has been suggested that this effect of oxygen tension on trophoblast differentiation and proliferation is mediated by TGFβ3 (77). Similarly, TGFβ3 induced by HIF-1α prevents scarring in fetal wound healing (78).

Stimuli for HIFs in RA

RA is a chronic inflammatory disease that is characterized by the formation of a hyperplastic synovium consisting of a variety of cell types, including synovial fibroblasts, macrophages, as well as B and T cells. During the course of the disease, the hyperplastic synovium invades deeply into cartilage and bone, leading to progressive destruction of the affected joints. Blood vessels are abundant in the RA synovium, and the total number of capillaries is increased compared with that in normal synovium (79). Moreover, morphometric studies have shown that capillary density appears to be increased in the RA synovium (80). This issue is controversial, however, because, due to an increased distance between individual cells and the nearest blood vessel in hyperplastic synovium, the capillary density might appear to be decreased in certain residues (81,82). In addition to the possible decrease in vascular density, the vascular system within RA synovium is less well organized, thereby disturbing the uniform perfusion of the tissue. Thus, despite the increase in the absolute number of vessels in the RA synovium, there is a relative hypoperfusion and hypoxia, particularly in the lining layer (80,83). Moreover, the gain of synovial fluid in affected joints of patients with RA increases the intraarticular pressure, leading to a further reduction of the perfusion (84). This effect is obvious in the resting joint and becomes even more obvious during movement of the affected joint (85). During exercise, synovial capillaries collapse because of the additional increase in intraarticular pressure. After reopening of the capillar-

ies, the intraarticular pressure returns to basal levels. This mechanism not only may lead to a further aggravation of local hypoxia, but also induces the expression of proinflammatory mediators in the joint in a hypoxia/reoxygenation-dependent manner (86).

The presence of reduced oxygen levels in the RA synovium is further supported by direct measurements of the oxygen tension and more indirectly by an increase of hypoxic metabolites in the synovium (87). Analyses of the oxygen tension in synovial fluid demonstrated that the mean oxygen levels in patients with RA are reduced to less than half of those in healthy controls (27 mm Hg and 63 mm Hg, respectively). Interestingly, the levels of synovial fluid Po_2 correlated with the histologic severity of the disease, in that patients with severe disease showed lower levels of Po_2 and vice versa (88,89). Direct measurement of the oxygen tension in the synovium of RA patients and healthy controls with a polarographic needle electrode confirmed the reduction of the oxygen tension in the RA synovium compared with that in normal synovium (90).

For a variety of cells, glycolysis is the most important means of generating ATP in the absence of oxygen, and, in fact, a shift to glycolytic metabolism is a long-known feature of RA synovium (91). Other markers of a hypoxic metabolism are also increased in RA synovium; e.g., levels of lactate as well as acidosis are enhanced (89).

In addition to hypoxia, a variety of growth factors and cytokines are up-regulated in RA and are able to stabilize and activate HIF-1α. Included are well-characterized key players in the pathogenesis of RA such as IL-1β and TNFα. Stimulation of cultured synovial fibroblasts with recombinant IL-1β and TNFα increases levels of HIF-1α mRNA. Moreover, incubation with IL-1β leads to stabilization of HIF-1 (92). Notably, the impact of other growth factors on HIF-1 has not been addressed specifically in RA.

HIFs in RA

Despite circumstantial evidence for the presence of hypoxia in the RA synovium, surprisingly little is known about the role of the HIF family members in the pathogenesis of RA. Hollander et al analyzed the expression of HIF-1α in RA synovium by immunohistochemistry (93). The levels of HIF-1α were strongly increased in RA samples compared with osteoarthritis (OA) samples, and no signal was observed in healthy controls. HIF-1α was expressed in the lining layer and to a lower extent in the sublining. Staining of serial sections with anti-CD68 antibodies revealed that HIF-1α was mainly expressed by synovial macrophages. The up-regulation of HIF-1α protein in RA synovium compared with normal synovium was confirmed in another study (94). However, strong expression of HIF-1α as well as HIF-2α was also found in synovial specimens obtained from patients with OA. In addition, HIF-1α and HIF-2α were detected in a variety of cell types, including synovial fibroblasts, macrophages, and endothelial cells (94).

In a recent landmark study (95), Cramer et al demonstrated that HIF-1α is essential for the initiation and perpetuation of myeloid cell–mediated inflammation. Mice with a specific deletion of HIF-1α in myeloid lineage cells developed less severe disease in different models of acute and chronic inflammation as compared with wild-type mice. These included a model for arthritis, in which arthritis is passively induced by transfer of pooled serum from K/BxN T cell receptor transgenic mice. Interestingly, these effects were independent of VEGF, and were attributable to severely reduced glycolysis and energy generation in myeloid cells. Consequently, energy-dependent processes such as intracellular killing of bacterial pathogens, homotypic adhesion, Matrigel invasion, and motility were significantly impaired in HIF-1α knockout macrophages (95).

Interactions between stromal cell–derived factor 1 (SDF-1; CXCL12) and its receptor CXC chemokine receptor 4 (CXCR4) provide another link between hypoxia signaling and inflammation. SDF-1 is a chemokine of the CXC family that promotes migration and activation of CD4 memory cells and other cells expressing CXCR4. It was shown recently that SDF-1 was strongly induced in synovial tissue explants as well as in cultured synovial fibroblasts under hypoxic conditions, whereas various inflammatory cytokines such as TGFβ, IL-1β, and TNF had no effect (96). SDF-1 is expressed in high levels in the lining layer and in perivascular areas of the sublining of patients with RA (97,98). In contrast, SDF-1 is detectable only in single cells in the lining of OA patients and healthy controls, consistent with the higher oxygen tension in these joints. In addition, the percentage of CXCR4-positive CD4+ memory cells is significantly elevated in the RA synovium compared with that in OA control synovium. Interestingly, almost all CD4+ memory cells in the RA synovium express CXCR4, although only one-third of peripheral CD4 memory cells from these patients express this receptor. Therefore, one might speculate that CXCR4-positive CD4+ memory cells migrate along a gradient of SDF-1 into the synovium, suggesting an important role for hypoxia-induced SDF-1 in the accumulation of memory

cells in the synovium of RA patients. In a similar manner, SDF-1 appears also to stimulate the migration of monocytes into the synovium, as shown with U937 cells in a SCID mouse transplantation model (99). The importance of the SDF-1/CXCR4 pathway in the pathogenesis of inflammatory arthritis is emphasized by findings in collagen-induced arthritis in mice (100). Treatment of the mice with AMD3100, a specific antagonist of CXCR4, between the time of immunization and development of the first symptoms of arthritis, reduced infiltration of leukocytes, hyperplasia of the synovium, and pannus formation.

The role of VEGF in RA

Angiogenesis has been suggested as a crucial pathogenic step in early RA (101). Indeed, endothelial cells in the RA synovium are activated, showing an increased expression of proliferation markers such as proliferating cell nuclear antigen and Ki-67 compared with that in patients with OA and healthy controls (102). Furthermore, synovial fluids from patients with RA induce the formation of tubular networks resembling capillaries in cultured endothelial cells (103). These findings demonstrate an up-regulation of angiogenesis in the diseased joint, but the formation of new vessels fails to keep pace with the rapid thickening of the synovium, largely caused by hyperplasia through the influx of macrophages and the impaired apoptosis of RA synovial fibroblasts. In addition, markers of cell death are increased in endothelial cells of RA patients, which suggests increased endothelial cell turnover in the inflamed synovium (104). The stimulation of angiogenesis under hypoxic conditions is part of the long-term response in order to reestablish normal tissue oxygen supply. This situation is similar to that observed in rapidly growing tumors. The high metabolic demand of the proliferating tumor cells results in hypoxia, especially in the inner parts of the tumor, which in turn stimulates angiogenesis to enhance the supply of nutrients (105).

VEGF is one of the strongest and best characterized stimulators of angiogenesis (for review, see refs. 106 and 107). Hypoxia up-regulates levels of VEGF mRNA by transcriptional activation via HIF-1α and, to a certain extent, by an increase in mRNA stability. VEGF is involved in the regulation of different steps of the angiogenic cascade. As such, VEGF contributes to the initial vasodilatation by induction of nitric oxide, and increases the permeability of endothelial cells, but it also induces synthesis of plasminogen activators and matrix metalloproteinase 1 in endothelial cells necessary for remodeling of the perivascular matrix. Furthermore, VEGF prevents endothelial cells from undergoing apoptosis and stimulates the proliferation and migration of endothelial cells (16,43). Application of VEGF as a single factor is able to induce angiogenesis in in vivo models such as the cornea pocket assay and the chorioallantoic membrane assay.

Levels of VEGF are markedly higher in the serum as well as the synovial fluid of patients with RA compared with patients with OA and healthy controls (108,109). The concentration of VEGF in the serum of patients with RA correlates well with the levels of C-reactive protein (110). In addition, there is also a significant correlation between serum levels of VEGF at early stages of RA and the development of radiologic deterioration within 12 months, as analyzed by radiographs scored according to the modified Sharp method (111). Consistent with these findings, high vascularity detected by dynamic enhanced magnetic resonance imaging or power Doppler ultrasonography is correlated with increased joint erosion in early RA (112). The important role of HIF-1α/hypoxia-induced VEGF synthesis in the pathogenesis of RA is further supported by a study in which a soluble form of VEGF receptor 1 was applied to mice with collagen-induced arthritis (113). Soluble VEGF receptor 1 functions as an antagonist of VEGF signaling, and treatment with this molecule significantly reduced paw swelling and joint destruction in this model.

Open questions

Based on our ongoing interest in the effects of hypoxia in various systems (8–10,25–27,32,77,78,114) and in particular in the rheumatic diseases (106,107,115–118), we aimed to discuss notions relevant to RA. In this regard, these findings outline the possible role of hypoxia and HIF-1α in the pathogenesis and progression of RA. They might also offer a chance for new, therapeutic strategies in the treatment of RA. Based on the circumstantial evidence for reduced levels of oxygen in the diseased joints, such strategies include the generation of "mobile" cells such as monocyte/macrophages, which are transduced with therapeutic genes under the control of a hypoxia-inducible enhancer (93). Application of such cells would then lead to selective expression of the gene of interest in the hypoxic joints and would thereby allow targeting of multiple joints at the same time. However, many problems have to be solved before such a scenario might become applicable for use in the clinic.

Genes under the control of a hypoxia-inducible promoter are likely expressed not only locally in the affected joint after systemic application, but also in a variety of other physiologically hypoxic tissues, giving way to a number of unwanted effects (8,9,114). In addition, many molecular aspects of HIF-1 signaling have not yet been addressed in RA. For example, to date, downstream pathways of HIF-1α have not been analyzed systematically and might include molecules favoring as well as antagonizing certain pathways of the disease. Other open questions include whether hypoxia and signaling via HIFs might contribute to the aggressive and invasive phenotype of synovial fibroblasts as observed for tumor metastases, and whether RA synovial fibroblasts might react differently to hypoxia than do normal fibroblasts or fibroblasts from other organs. Furthermore, the expression of HIF-2α and HIF-3α in the joint has not been investigated. Are they also up-regulated in RA synovium as has been demonstrated for HIF-1α? If so, are there links for HIF-2α and HIF-3α to the pathogenesis of RA?

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